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Doxorubicin Enhances Curcumin's Cytotoxicity in Human Prostate Cancer Cells In Vitro by Enhancing Its Cellular Uptake

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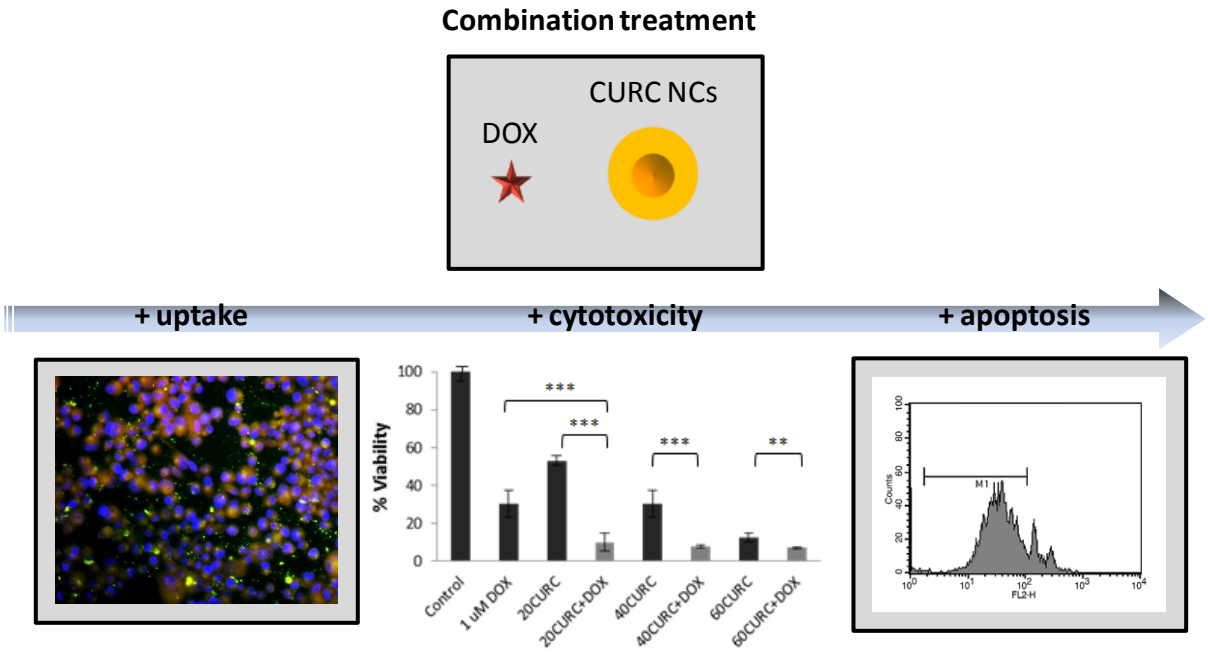
Graphical abstract



Abstract

Doxorubicin (DOX) is a widely used drug in cancer treatment. Despite its popularity, it suffers from systemic side effects and susceptibility to drug resistance. Curcumin (CURC), on the other hand, is a drug that recently gained popularity due to its wide range of biological activities, including anti-inflammatory and anti-cancer activities. Limitations to its clinical translation include its poor water solubility and the need for administration of high doses. Combinatory anti-cancer therapy has been proposed as a common approach to overcome one or more of these challenges. In this work, we propose a combinatory DOX and CURC anti-cancer therapy of prostate cancer cells *in vitro*. DOX and CURC were administered in the free drug and nanocapsule form, respectively. Cell size and complexity, cytotoxicity and apoptosis were studied by flow cytometry, MTT assay and sub-G1 quantification, respectively. Cellular uptake of CURC nanocapsules (CURC NCs) was quantified with by fluorescence microscopy and high-performance liquid chromatography fluorescence

detection. Results showed that *in vitro* treatment with CURC NCs in the presence of subtherapeutic concentrations of DOX, led to significant increase in prostate cancer cells (PC3) apoptosis and death. This was likely due to significantly enhanced CURC uptake by the cells. The study presents a good rationale for pursuing combinatory CURC/DOX therapy in pre-clinical tumor animal models in the near future.

1. Introduction

Cancer treatment options can be limited due to multidrug resistance and side effects. Recently, combination chemotherapy of multiple anticancer drugs has been extensively developed for overcoming these major pitfalls (Li et al., 2014). The combinational anti-cancer therapy of doxorubicin (DOX) and curcumin (CURC) represents an attractive strategy over single drug treatment to maximize the therapeutic response. DOX is a broad-spectrum chemo-therapeutic agent widely used for the treatment of several cancers including breast, ovary, cervix and prostate as reviewed recently (Tacar et al., 2013). Its effectiveness can be limited due to its high toxicity and side effects, including myelosuppression, alopecia, acute nausea, vomiting, stomatitis, cumulative cardiotoxicity (Hortobagyi, 1997), and multidrug resistance resulting, after repeated administration (Shen et al., 2008). Interestingly, a phase II trial showed that liposomal doxorubicin led to only modest anticancer activity for the treatment of hormone-refractory prostate cancer and it was suggested to include this agent in combination chemotherapy (Harris et al., 2002). In clinic, DOX has been used in multi-drug regimens with other anti-cancer agents, such as cyclophosphamide, 5-fluorouracil, docetaxel, vinblastine, and bleomycin (Hernandez and Perez, 1996; Itoh et al., 2000; Martin et al., 2003) Recent studies have reported that curcumin (CURC) can reduce DOX's adverse reactions (Sadzuka et al., 2012).

CURC has been reported in many studies to delay tumor growth *via* modulating different signalling pathways. Its limitations include poor water solubility and low potency, requiring administration of high CURC doses, in a solubilized drug form (Anand et al., 2007). We have recently reported the preparation of CURC NCs and confirmed their therapeutic efficacy in colon-bearing mice, following intravenous administration (Klippstein et al., 2015).

In this study, we propose a combinatory anticancer approach with free form and nanoformulation of DOX and curcumin, respectively. We hypothesize that co-treatment of human prostate cancer cells with CURC NCs and subtherapeutic doses of DOX can improve CURC's uptake in cells improving its therapeutic efficacy. *In vitro* cellular uptake and drug quantification studies, followed by cytotoxicity and apoptotic assays were carried out to test our hypothesis.

2. Material and Methods

2.1 Material

75/25 DL-lactide/glycolide conjugate (PLGA), Mw ≈ 18000, was a gift from Purac Biomaterials. CURC was purchased from Santa Cruz Biotechnology (UK). Snake Skin dialysis tubing (MWCO 10,000 Da) was purchased from Thermo-fisher (USA). Soybean lecithin (Epikuron 140 V) was a kind gift from Cargill Pharmaceuticals. Castor oil, Tween® 80, acetone and absolute ethanol were obtained from Sigma-Aldrich (UK). RPMI-1640 media, foetal bovine serum (FBS), penicillin/streptomycin, trypsin/EDTA, and phosphate buffered saline (PBS) were obtained from Gibco, Invitrogen (UK).

List of chemical compounds

PLGA (PubChem CID: 23111554); Soybean lecithin (PubChem CID: 57369748); CURC (PubChem CID: 969516); castor oil (PubChem CID: 14030006); Tween® 80 (PubChem CID: 5281955); ethanol (PubChem CID: 702); acetone (PubChem CID: 120).

2.2 Cell Culture

The human prostate carcinoma cells DU145 (ATCC® HTB-81™) and PC3, kindly provided by Dr. M. Japon; Department of Endocrine Tumorigenesis and Hormonal Regulation of

Cancer, Biomedicine Institute of Seville, IBIS, CSIC-University of Seville, Spain, were cultured in Advanced RPMI media supplemented with 10% FBS, 50 U mL^{-1} penicillin, 50 µg mL^{-1} streptomycin, 1% l-glutamine, at 37 °C in 5% CO_2. Cells were routinely grown in 75 cm^2 canted-neck tissue culture flasks and passaged twice a week using Trypsin/EDTA at 80% confluency.

2.3 Formulation of the NCs

CURC NCs were prepared using the nanoprecipitation technique as described in one of our previous studies (Klippstein et al., 2015). Briefly, CURC (5 mg), soybean lecithin (25 mg), and PLGA polymer (25 mg), were mixed at 1:2:0.1 molar ratio with 300 µL of castor oil dissolved in 5 mL of acetone/ethanol (60:40 v/v) mixture. This organic phase was added dropwise into the aqueous phase (10 mL) containing Tween® 80 (0.2%) as a hydrophilic surfactant; the mixture was maintained under magnetic stirring in the chemical hood for 30 min to allow solvent to diffuse and form NCs. Organic solvents were then eliminated by evaporation under reduced pressure using a Buchi rotavap. The final volume of the colloidal suspension was adjusted to 10 mL.

2.4 Uptake Studies In Vitro by Flow Cytometry

Cells were seeded at a density of 5×10^4 in 24-well plates, allowed to attach overnight and then treated with 20 µM of CURC NCs and 1 µM of DOX. After treatment, the cells were washed twice with PBS, trypsinized and centrifuged at 1500 rpm for 5 min and the cell pellet was resuspended in 250 µL of PBS. The internalization of CURC and DOX was studied on 10,000 gated cells by detecting the fluorescence using FL2 channel detector and BD FACS Calibur flow cytometer (BD Biosciences). The measurements were done in triplicate and presented as mean ± SD.

2.5 Uptake Studies In Vitro by Confocal Laser Scanning Microscopy

PC-3 cells were seeded onto glass coverslips at a density of 25 K cells per a well of a 24-well plates in RPMI media overnight. Cells were then incubated with 0.5 mL of 20 μM CURC NCs or 1 μM DOX or the combination for 4 h. At the end of incubation period, cell were rinsed, fixed (200 μL of 4% PFA for 15 min at RT) and subsequently counterstained with DAPI. Coverslips were mounted on glass slides using VectaShield mounting media. CURC NCs uptake was represented by the green signals, while DOX was represented by red signals and nuclei were detected in blue. Confocal images were captured using a Nikon Eclipse Ti inverted microscope, Nikon, Tokyo, Japan, using an objective lens 20×/0.75NA 505 nm output filter, and a Plan-Neofluar 10 lens.

2.6 Quantitative Determination of Curcumin Uptake In Vitro by HPLC-FLd

CURC uptake was quantified using a high-performance liquid chromatography fluorescence detection (HPLC-FLd) analytical method. Method validation has been reported in our previous study (Klippstein et al., 2015). Approximately $5x10^5$ PC3 cells were treated in a 6-well plate with 1 μM DOX, 20 or 40 μM of CURC NCs or the combination for 4 h and 6 h. Cells were transferred into 15 mL test tubes and vortex-mixed for 20 s. One millilitre of 1X PBS was added and samples were homogenized using an ULTRA-TURRAX T18 Homogenizer (IKA, GE). After the tissue was completely homogenized, 2 mL of the extraction solvent consisting of 98% acetonitrile and 2% TFA (v/v) was added and were left under agitation overnight. Samples were then centrifuged at 3000× *g* for 5 min at 4 °C and 1 mL of the supernatant was transferred to a clean glass tube. The extraction was repeated twice with 1 mL of extraction solvent, and the pooled supernatants were concentrated using a centrifugal evaporator Genevac EZ2 (Genevac Ltd, UK) at low heat setting (40 °C). The content was transferred to an injection sample vial, and 50 μL was injected into the HPLC-

FLd system (Agilent Technologies) using a Phenomenex C18 column (150 × 2.1 mm, 3.6 μm) and a flow rate of 0.2 mL min. The mobile phase consisted of A: 0.1% TFA in H_2O: acetonitrile (90:10) (v/v) and B: 0.1% TFA in H_2O: acetonitrile (10:90) (v/v). The elution gradient was 10%–90% B in 30 min. Fluorescence detection, excitation 345 and emission 530 nm. For HPLC-FLd analysis, CURC working solutions were prepared by dilution of the stock solution with the mobile phase reaching final concentrations of 1.0, 2.0, 4.0, and 8.0 μg mL −1. Peak areas were plotted against CURC concentrations. The linearity of the detector was determined over the concentration of CURC and a standard curve was calculated by linear regression.

2.7 In Vitro Cytotoxicity Studies

PC-3 cells were seeded in 96-well plates and incubated with different concentrations of NCs or DOX (200 μL final volume) in complete media for 24 and 72 h. Cytotoxicity was examined by MTT assay. Briefly, at the end of the incubation period, media was removed and replaced with 120 μL of MTT solution at a final concentration of 0.5 mg mL^{-1}. Cells were incubated for 3 h at 37 °C and 5% CO_2. At the end of the incubation, formazan was dissolved in 200 μL of DMSO and the plate was read at 570 nm in a FLUO star OPTIMA plate reader (BMG Labtech). Results were expressed as the percentage cell survival (mean ± SD) and calculated using the following equation: % Cell survival = (A570 nm of treated cells/A570 nm of untreated control cells) × 100.

2.8 Assessment of Late Apoptosis by Sub-G1 Quantification

Late apoptosis induced by CURC NCs and/or DOX were measured by quantifying the fraction of cells displaying reduced amount of DNA, named sub-G1 population. PC-3 and DU-145 cells were seeded at 5×10^4 cells per well in 24-well plates and incubated overnight

at 37 °C with 5% CO_2. Cells were then treated with 20 μM and 40 μM of CURC NCs. At the end of treatment period, floating cells were collected and adherent cells were trypsinized and transferred into BD flow cytometer tubes. After centrifugation at 800 × g for 5 min and washing in PBS, cells were re-suspended in 100 μL of PBS and 900 μL of 70% cold ethanol for fixation and stored overnight at 4 °C. Fixed cells were then centrifuged, washed in PBS, and treated in a mix of 250 μL of PBS and 250 μL of DNA extraction buffer (Na_2HPO_4 190 × 10^{-3} m, Citric Acid 4 × 10^{-3} m, pH 7.8) for 10 min at 37 °C. Samples were then centrifuged and re-suspended in 50 μL of RNase in PBS (100 μg mL^{-1}) before being stained for 30 min at 37 °C in the dark with 400 μL of propidium iodide (PI) solution (40 μg mL^{-1} of PI in PBS). PI fluorescence was analyzed by flow cytometry using a BD FACS Calibur flow cytometer obtained from BD Bioscience (US). Finally, 10,000 cells were gated and fluorescence of Sub-G1 population was analyzed in triplicates for each condition using the FL2 detector. Results were expressed as average ± SD (n = 3).

3. Results

3.1 Physico-chemical Characterization of CURC NCs and CURC loading.

CURC NCs formulation and physico-chemical characterization have been described in our previous report (Klippstein et al., 2015). In brief, CURC NCs exhibited hydrodynamic diameter and zeta-potential of 150.5 ± 4.7 nm and -37.2 ± xx mV, respectively. Percentage encapsulation efficiency and drug loading were 92.3% ± 1.6 and 18.4% + 0.3, respectively.

3.2 Morphological studies of PC3 cells by flow cytometry

The morphology of PC3 cells was evaluated after treatment with DOX, CURC NCs and the combination. The cells were treated for 4 h and size (FSC) and granularity (SSC) were measured by flow cytometry. Cells treated with the combination became less granular and significantly smaller in size compared to cells treated with either DOX or NCs (**Figure 1**). This morphology is typically associated with dying cells suggesting an additive effect of both drugs.

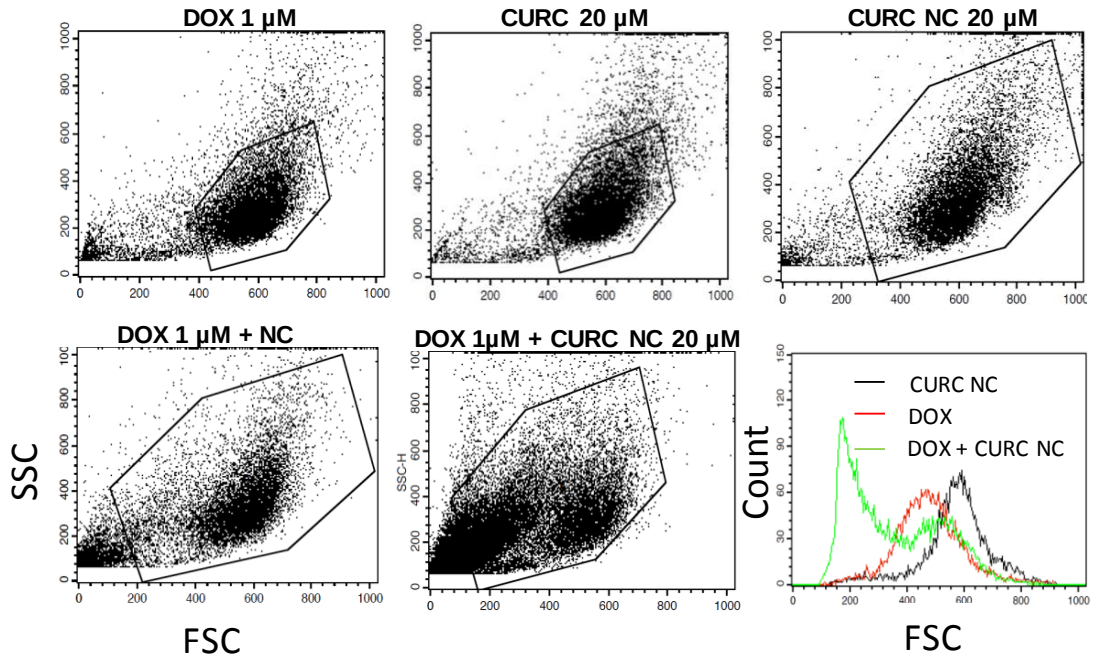


Figure 1. Cell size and granularity of PC3 prostate cancer cells, after intracellular uptake of doxorubicin (DOX), empty NC (NC), curcumin-loaded NC (CURC NC) or their combinations. Changes in cell size (FSC detector) and granularity (SSC detector) were assessed after 4 h of incubation by flow cytometry. The combination of DOX and CURC NC but not any of the individual treatments lead to reduction in cell size, reflecting enhanced overall cytotoxicity of the combinatory treatment.

3.3 Intracellular uptake studies of DOX, CURC NCs and the combination in PC3 human prostate cancer cells in vitro.

The intracellular uptake of intrinsically fluorescence CURC and DOX was studied in PC3 cells by fluorescence microscopy, flow cytometry and HPLC-FLd. PC3 cells were treated with 1 µM DOX and 20 or 40 µM of CURC NCs for 4 h. The cellular uptake and distribution of DOX and CURC was investigated by fluorescence microscopy (**Figure 2A**). As DOX and CURC exhibit intrinsic green and red fluorescence, respectively, no additional probe labelling, except DAPI to stain the nuclei, was required for imaging. At 4 h, DOX primarily located in the nuclei; whereas CURC NCs were detected in the cytoplasm. This can be verified by the fact that the nuclei counterstained with DAPI (blue color) were less evident for DOX treatments than for CURC NCs. The uptake of the combination treatment was studied by flow cytometry (**Figure 2B**). Cells that appeared smaller in size and with lower granularity were gated separately (gate 2), showing two fluorescence peaks matching CURC and DOX peaks. In contrast, cells that appeared larger in size and with higher granularity (gate 1) only showed one fluorescence peak corresponding to DOX. This result indicates that DOX is taken up by most of the cells, within 4 h treatment, and that only those that have taken up CURC NCs as well as DOX, start shrinking as a consequence of cell-death. Additionally, a higher uptake of CURC NCs is detected when cells were co-treated with DOX. This suggests that DOX helps NC internalization into cells.

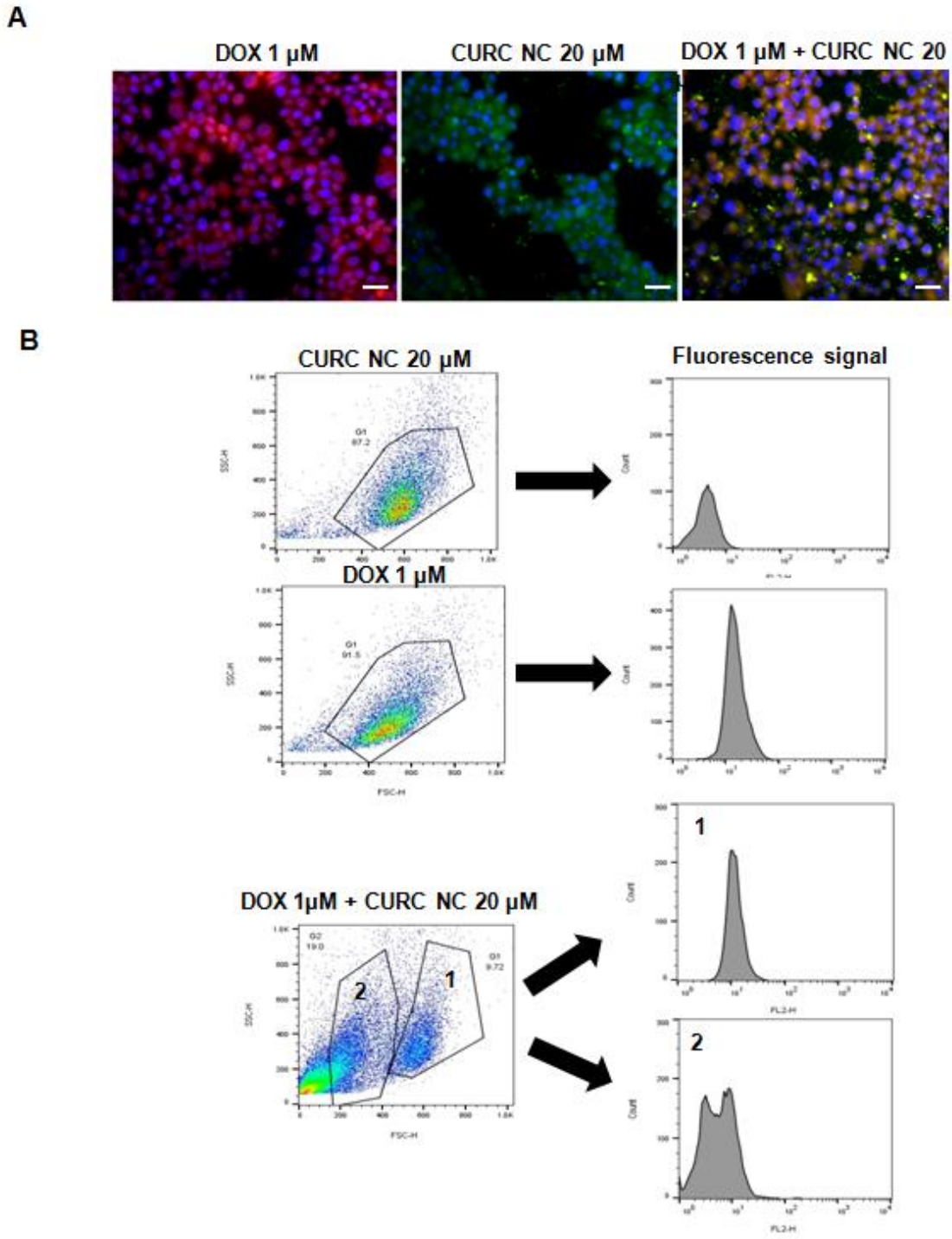


Figure 2. Intracellular uptake of CURC NC, in presence or absence of DOX, in PC3 human prostate cancer cells *in vitro*. (A) Cellular uptake of DOX (red fluorescence) and CURC NC (green fluorescence) was assessed by fluorescence microscopy after 4 h of incubation. Nuclei were counterstained with DAPI (blue). The scale bar corresponds to 25 µm. **(B)** Dot plots showing PC3 cells after 4 h of incubation with the different treatments. Histograms display the fluorescence signals detected from each cell population. Cells treated with CURC NC or DOX show individual cell population; with low and high FL2 intensity signals, indicative of CURC and DOX uptake, respectively. Cells treated with the combinatory treatment show two overlapping populations; cells in population 1 are larger in size and are positive for DOX signals (high FL2 intensity). Cells in population 2 are smaller in size, reflecting cytotoxicity, and are positive for CURC signals (low FL2 intensity).

To confirm this observation, the uptake of CURC NCs in PC3 cells, in absence or presence of DOX, was quantified. Cells were treated with DOX, CURC NCs or the combination for 4 h and 6 h. The cells were then lysed and fluorescence intensity was quantified by HPLC-FLd. Commercially available CURC consists of curcumin, demetoxycurcumin, and di-demethoxycurcumin. These were observed at retention times of 21.2, 21.8, and 22.5 min **(Figure S1)**. CURC was quantified using a calibration curve calculated by linear regression **(Figure S1)**. Interestingly, CURC fluorescence detected for the combination treatment DOX (1 µM) and CURC NCs (40 µM) was higher than the equivalent doses of CURC NCs alone **(Figure 3C)**. This confirms our hypothesis that an enhancement in CURC NCs uptake has occurred when cells were co-treated with DOX.

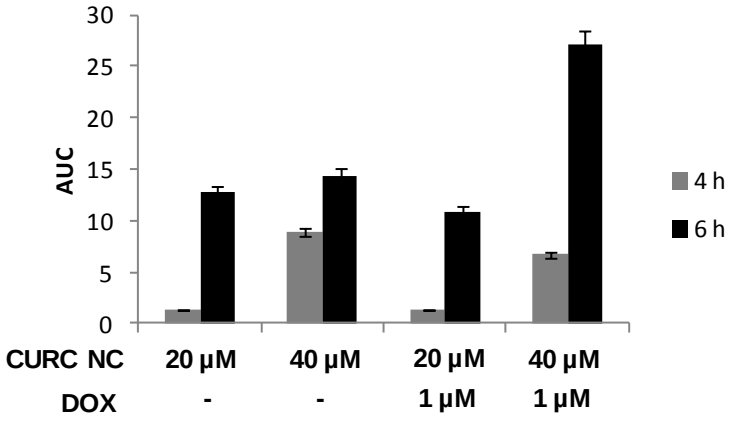


Figure 3. Quantification of the intracellular uptake of CURC NCs in PC3 human prostate cancer cells *in vitro* by HPLC-FLd. Cells were quantified for intracellular CURC content following 4 h or 6 h of incubation by HPLC-FLd, using a C18 column, excitation/emission wavelengths of 345/530 nm and 0.1% TFA in H_2O: acetonitrile mobile phase.

3.4 CURC NCs potentiates DOX-induced cytotoxicity of human prostate cancer cells in vitro.

The cytotoxic effect of DOX, CURC NCs and the combination was screened in PC3 cells *in vitro*. Light microscopy images showed no morphological changes when cells were treated

with DOX or CURC NC for 24 h. As expected, cells treated with the combination appeared more round and smaller in size (**Figure 4A**). Cell viability was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 0.1 or 1 µM of DOX, 20 or 40 µM of CURC NC, or the combinations. An enhanced cytotoxicity was observed after 72 h incubation with the combination treatments at both 0.1 and 1 µM DOX and 20 and 40 µM of CURC NCs concentrations. The difference was moderate in the case of 0.1 µM DOX but increased dramatically in case of 1 µM of DOX **(Figure 4B)**.

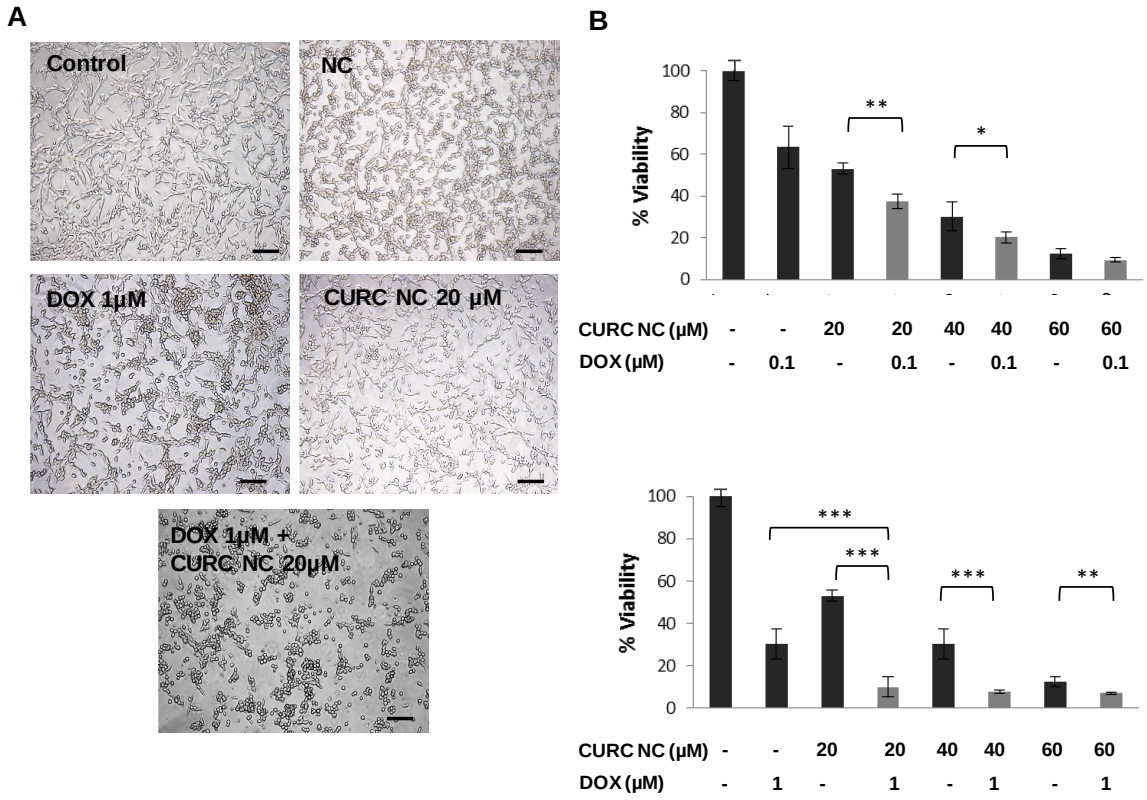


Figure 4. Cytotoxicity studies of CURC NC, in presence or absence of DOX, in PC3 human prostate cancer cells *in vitro*. (A) Microscopic examination of PC3 cells after treatment with DOX, CURC NC or combinatory treatment, for 24 h. Cell rounding and reduction in cell number was observed for cells treated with the combinatory treatment. The scale bar corresponds to 50 µm. **(B)** PC3 cells were incubated with DOX (0.1 or 1 µM), CURC NC (20, 40 or 60 µM) or the combinations for 72 h. Cell viability was determined by MTT assay. Values are expressed as mean ± SD (n = 3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA test). Asterisks correspond to statistically significant differences between particular groups.

3.5 CURC-loaded NCs and DOX combinations enhanced the apoptosis in human prostate cancer cells.

Sub-G1 analysis allows quantification of hypodiploid cell percentage entering into the apoptotic SubG1 phase. PC3 cells and DU145 cells were analyzed by FACS and results showed that DOX combined with CURC NCs treatments increased SubG1 population of cells at 24 h. Interestingly, the combination treatment of 1 µM DOX and 20 µM CURC NCs showed a 20% significant increase in the subG1 phase. Similar values were observed with higher concentrations of DOX and CURC NCs, confirming that 1 µM DOX and 20 µM NCs seem to be the most effective for cell killing.

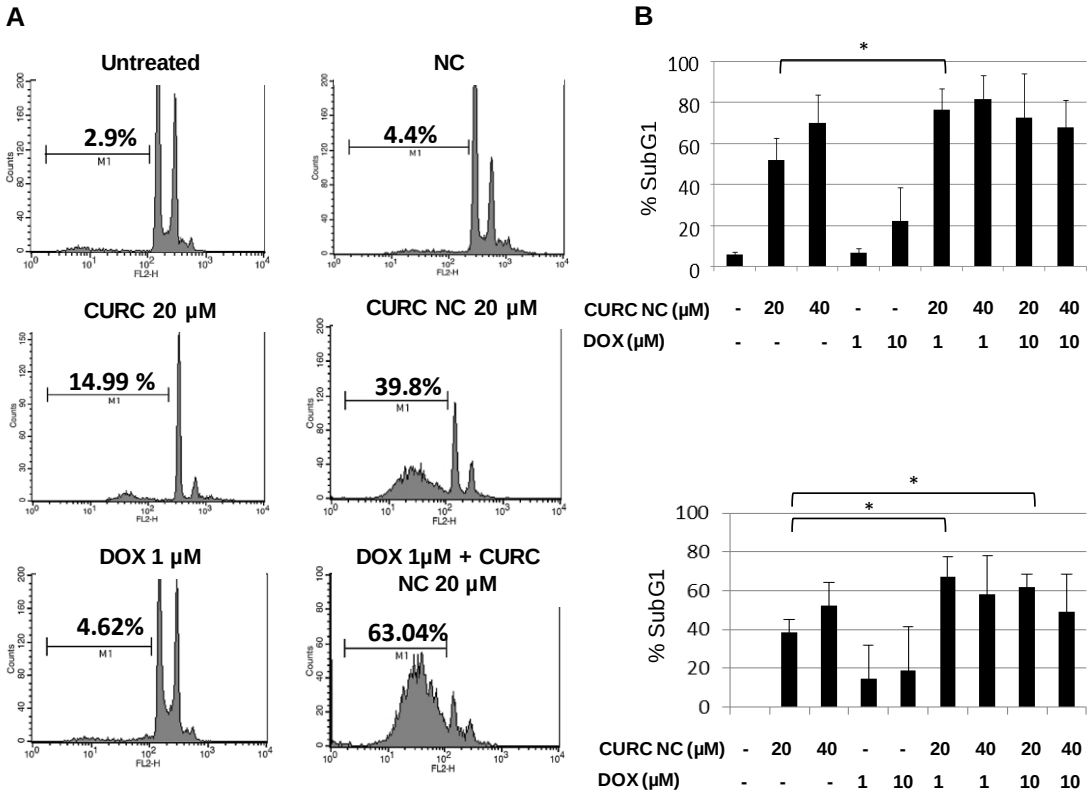


Figure 5. Percentage SubG1 cell population in PC3 human prostate cancer cells following combinatory treatments *in vitro.* (A) Representative histograms of SubG1 quantification in PC3 cells incubated with DOX, CURC, CURC NC or the combinatory treatments, following 24 h treatment. **(B)** Percentage SubG1 quantification in PC3 (top) and DU145 cells (bottom) after 24 h treatment. SubG1 was quantified by using PI staining method. The combinatory treatment significantly increased the % of SubG1, compared to individual DOX or CURC treatments. Values are expressed as mean ± SD (n = 3).

4. Discussion

DOX is one of the most widely used therapeutic drugs in cancer. Unfortunately, many tumor cells are inherently resistant to DOX or can acquire chemo-resistance shortly after commencing the therapy, leading inevitably to treatment failure (Notarbartolo et al., 2005). An effective strategy to tackle chemo-resistance is the identification and evaluation of drug combinations (Misra and Sahoo, 2011). Previous studies have demonstrated that CURC may interfere with NF-kB activation and increase tumor cell response to different NF-kB activating anticancer drugs, including DOX (Hour et al., 2002; Notarbartolo et al., 2005). Its major roadblocks for clinical translation in cancer therapy are however related to its poor water solubility, poor pharmacokinetics and limited bioavailability (Kunnumakkara et al., 2008). In a previous report, we have shown that long blood-circulating CURC-loaded oil-cored polymeric NCs delivered doses of CURC to solid tumors, after systemic administration *in vivo* (Klippstein et al., 2015), that were sufficient to result in tumor (colon) growth delay. In an attempt to improve the therapeutic efficacy of CURC, a combinatory anti-cancer therapy approach is proposed here by using low doses of DOX (in free form) and s form of CURC. The rationale for using free DOX here was that the free drug can enter the cell very quickly by passive diffusion (Soininen et al., 2016) which then facilitates entry of CURC NCs that is otherwise only effective at high doses. This combination therapy approach may offer some advantages over other nanoformulations co-encapsulating both drugs in the same carrier. Their rationale was that co-encapsulation of DOX and CURC in one carrier could lead to an interaction of both drugs and may result in an incomplete release (Zhao et al., 2015). In addition, other reports have demonstrated a reduction in DOX cytotoxicity when encapsulated in a nanoformulation (Gonzalez-Fajardo et al., 2016).

It is unclear why co-treatment with DOX improves the therapeutic efficacy overall. The intracellular uptake of DOX, CURC NCs and the mixture was studied by fluorescence

microscopy, flow cytometry and HPLC-FLd. Interestingly, fluorescence microscopy, flow cytometry and HPLC-FLd all showed complementary results that best results were achieved when cells were treated with the mixture of 1 μM DOX and 40 μM CURC NCs at 6 h and suggest that co-treatment with DOX led to an increase in intracellular uptake of CURC NCs. There was a clear reduction in cell size, reflecting an enhanced toxicity compared to single treatments. Low concentrations of DOX can result in loss of membrane integrity (Eom et al., 2005) facilitating entry of the rather normally "slow-entering" NCs. Another possible explanation, which cannot be excluded here, is that CURC could help retaining DOX in the cell nucleus and inhibit its efflux, as supported by others (Misra and Sahoo, 2011).

5. Conclusion

This work reports that combinatory treatment of DOX and CURC NCs can improve the outcome of anti-cancer therapy in prostate cancer *in vitro*. While the exact mechanism is still unknown, we confirmed that the uptake of CURC NCs in cancer cells significantly improved upon co-treatment with DOX, at sub-therapeutic concentrations of the latter. Combination treatments enhanced significantly cell viability compared to single treatments, assessed by cytotoxicity and apoptosis studies in prostate cancer cells *in vitro*, particularly at 1 μM DOX and 20 μM CURC NCs combinatory treatment. Future studies will focus on testing therapeutic efficacy in prostate cancer tumor-bearing mouse models.

Acknowledgements

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Figure captions

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